Tactic-specific differences in seminal fluid influence sperm performance

Lisa Locatello, Federica Poli and Maria B. Rasotto

Department of Biology, University of Padova, Via U. Bassi 58/B, 35121 Padova, Italy

Seminal fluid often makes up a large part of an ejaculate, yet most empirical and theoretical studies on sperm competition have focused on how sperm characteristics (number and quality) affect fertilization success. However, seminal fluid influences own sperm performance and may potentially influence the outcome of sperm competition, by also affecting that of rivals. As a consequence males may be expected to allocate their investment in both sperm and seminal fluid in relation to the potential level of competition. Grass goby (Zosterisessor ophiocephalus) is an external fertilizer with guardsneaker mating tactics, where sperm competition risk varies according to the tactic adopted. Here, we experimentally manipulated grass goby ejaculates by separately combining sperm and seminal fluid from territorial and sneaker males. While sperm of sneaker and territorial males did not differ in their performance when they interacted with their own seminal fluid only, sperm of sneakers increased their velocity and fertilization rate in the presence of territorial males’ seminal fluid. By contrast, sneaker males’ seminal fluid had a detrimental effect on the performance of territorial males’ sperm. Sperm velocity was unaffected by the seminal fluid of males employing the same tactic, suggesting that seminal fluid’s effect on rival-tactic sperm is not based on a self/non-self recognition mechanism. Our findings show that cross interactions of sperm and seminal fluid may influence the fertilization success of competing ejaculates with males investing in both sperm and seminal fluid in response to sperm competition risk.

1. Introduction

Sperm competition, occurring whenever the sperm of rival males compete to fertilize the same group of eggs [1], is a widespread phenomenon and a powerful evolutionary force shaping male behaviour, morphology and physiology [2–4]. The most common adaptation to sperm competition in males is represented by an increase in sperm expenditure at mating to increase their probability of egg fertilization [2–5]. Indeed, comparative studies across both species and populations show that an increase in the level of sperm competition is paralleled by a greater ejaculate investment as judged by relative testis size, sperm number and sperm quality [2,6,7]. For example, in species in which male alternative reproductive tactics (ARTs) experience different levels of sperm competition, opportunistic males, playing the tactic associated with the higher level, release more sperm, which can be also faster and/or more viable, than males experiencing lower risk [8–12]. Moreover, males have been shown to rapidly adjust their ejaculate expenditure, in terms of sperm number and/or quality, when the level of sperm competition varies among successive matings, as well as in relation to social status and female quality [13–17].

To date, theoretical and empirical studies on the effects of sperm competition have primarily focused on sperm number and quality [2,18]. However, a substantial portion of the ejaculate is made up by the seminal fluid, which may indirectly influence paternity success by affecting female reproductive success [19]. Indeed, seminal fluid contains substances that decrease female receptivity, increase oviposition rate and form mating plugs [19,20], and males are capable of adjusting their amount in response to the perceived level of sperm competition [21]. Some studies have recently shown that seminal fluids may play a more direct role in sperm competition by affecting rivals’
sperm performance. Indeed, in promiscuous ants and bees seminal fluid incapacitates the sperm of rival males [22], while in other insects it improves equally the survival of own and other sperm [23,24]. This suggests that, unless a self/non-self recognition mechanism evolves [23], the function of seminal fluid to enhance own sperm performance can be exploited by the sperm of rival males [25].

Although the conditions for male parasitism of rival ejaculates are reasonably common in natural mating systems, the idea that males may gain fertilization advantage in allocating their seminal fluid investment in relation to mating order or role [25,26] still lacks experimental evidence, probably because it is difficult, in internal fertilizers, to attribute the seminal fluid to a specific individual. Here, we overcome this problem by using the grass goby, Zosterisessor ophiocephalus (Pallas), a fish species with external fertilization and guard-sneaker mating tactics [27]. In this species, territorial males, during the breeding season, dig and defend their nest, court females and perform parental care to the egg while sneakers parasitize the spawns of territorial males [27,28]. Territorial males release viscous ejaculates (sperm trails) on the nest ceiling, where eggs are laid both before and during egg deposition [27,28]. These ejaculates slowly dilute in seawater, thus releasing active sperm [27]. Sneaker males enter inside a nest when spawning occurs and release their ejaculate in proximity to those of territorial males and to eggs [28]. Sperm competition is intense, but in approximately 30 per cent of spawning, territorial males do not mate in competition while sneaker males always do [27]. Territorial males’ ejaculates contain more seminal fluid and fewer sperm than those of sneakers [27,28], but sperm quality, in terms of velocity, viability and adenosine-5’-triphosphate (ATP) content, does not vary with tactic (when assayed in a saline solution) [10]. It may be envisaged that in species with ARTs the seminal fluid of territorial males could be exploited by opportunistic males, to enhance the performance of their own sperm. Otherwise, territorial males’ seminal fluid could have a detrimental effect on sneaker’s sperm, to counteract their numerical superiority.

We tested these predictions by analysing whether seminal fluid affects performance, in terms of velocity and fertilization success, of own and rival sperm, according to the mating tactic employed by the male. We measured sperm performance by separating the sperm and seminal fluid components of ejaculates and making reciprocal combinations within and between males using different tactics. We also simulated the conditions of natural competition by using a mixture of the seminal fluids of sneaker and of territorial males.

2. Material and methods

(a) Animal sampling and handling

Males and females were collected in the Venetian Lagoon during their breeding season (April–June), and kept in separate tanks under artificial light (14 L:10 D). Water (20°C) was changed daily and fish were fed with fresh food. Each male was anaesthetized in a water solution of MS 222 (tricaine sulphate; Sandoz), his standard length (SL; distance between the snout and the base of the tail) was measured and his ejaculate was collected. Each male was categorized as territorial or sneaker on the basis of their size and the characteristics of their sperm trails, which are white in sneaker males, owing to the high sperm content, and dense and opaque in territorial ones, owing to the high mucin content and low amount of sperm [28].

(b) Gamete collection and ejaculate processing

Ejaculate was obtained through a gentle pressure on the abdomens of anaesthetized males and collected with a Gilson pipette. Ejaculate samples were centrifuged at 13 300g for 3 min at 4°C to separate sperm from the supernatant seminal fluid (mean fluid volume: territorial: 199 µl ± 123 s.d.; sneaker: 19 µl ± 16 s.d.). Sperm cells were then re-suspended in an extender inactivating medium (3.5 g l-1 NaCl, 0.11 g l-1 KCl, 0.39 g l-1 CaCl2, 1.23 g l-1 MgCl2, 1.68 g l-1 NaHCO3, 0.08 g l-1 glucose, pH 7.7) [29] and maintained at 3–5°C until analysis (within 1 h of collection). As the number of sperm varies among males and is significantly higher in sneakers than territorial males, the volume of inactivating solution was individually adjusted (range: sneaker, 70–800 µl; territorial, 45–500 µl), in order to standardize for sperm concentration in inactivated samples (76.069 ± 970 s.d. sperm µl-1). Sperm concentration was checked with an improved Neubauer chamber haemocytometer. Seminal fluid was also maintained at 3–5°C until analysis. Eggs were obtained from previously anaesthetized ready-to-spawn females, through a gentle pressure on their swollen abdomen, and collected on acetate sheets onto which they adhere. Immature eggs do not adhere well to the sheets, and thus these samples were easily detected and discarded. Acetate sheets with eggs were maintained in filtered seawater until the trials (within a few minutes of collection). All individuals were released, unharmed, at the site of collection.

(c) Sperm velocity measurement

Ten microlitres of sperm were taken from inactivated samples and activated by adding 20 µl of filtered seawater at 20°C ± 1°C, containing 2 mg ml-1 of bovine serum albumin. Activated sperm samples were then incubated for 2 min without seminal fluid or with 1.5 µl of different seminal fluid solutions (see §2e). In this species, eggs remain fertilizable for several hours and sperm are active for more than 30 min [10,27], thus 2 min of incubation represents a conservative time to guarantee they are not exhausted before performance measurements. Three microlitres of samples were then placed in separate wells on a 12-well multistest slide (MP Biomedicals, Aurora, OH) previously coated with 1 per cent polyvinyl alcohol (Sigma-Aldrich), to avoid sperm sticking to the glass slide [30], and covered with a coverslip. Sperm velocity was measured using a CEROS Sperm Tracker (Hamilton Thorne Research, Beverly, MA). Mean speed measurements were based on 76.31 ± 28.05 (mean ± s.d.) sperm tracks per sample. Among sperm velocity measures we focused our analyses on curvilinear velocity (VCL), as this measure is positively correlated with fertilization success in many external fertilizers [31,32].

(d) In vitro fertilization

For each male, subsamples of 10 µl of sperm were activated with the addition of 20 µl of marine filtered seawater and incubated for 2 min with 1.5 µl of different seminal fluids (see §2e). A volume of sperm solution containing 8 × 106 sperm cells was then taken, diluted to 50 µl with filtered seawater, and used for fertilization trials, which were performed by placing an acetate sheet with a pool of eggs collected from three different females on the bottom of a glass beaker containing 500 ml of filtered seawater. Eggs were pooled with the intent of minimizing the potential male-by-female interaction effects at fertilization [33]. A new group of three females was used for each male, while the same three females gave the pools of eggs for the different treatments performed on each male. The three batches of eggs from each female were distributed randomly among treatments with respect to collection order from female. Sperm were homogeneously deposited on the water surface with a Gilson pipette (distance from surface to bottom = 8.5 cm). After 15 min, the acetate sheet was extracted, gently washed and placed in a...
new glass beaker with clean filtered seawater and oxygen supply. The percentage of fertilized eggs of each pool was checked 4 h later, when one could clearly distinguish the complete lifting of chorion and the first stages of cellular division. For each trial, 273 ± 47 (mean ± s.d.) eggs were used.

(e) Experimental design

(i) Effect of seminal fluid on sperm performance

In a first experiment, we evaluated the effect of the seminal fluid, released by males performing different tactics, on sperm velocity. The sperm of each territorial male (n = 20; SL range: 13.7–18.5 cm) was tested after incubation in its own seminal fluid or that of a sneaker male. Conversely, the sperm of each sneaker male (n = 20; SL range: 6.4–9.3 cm) was incubated either in its own seminal fluid or in that of a territorial male. We also recorded sperm velocity after incubation with the seminal fluid of a male adopting the same tactic to further check if any effect of another male’s seminal fluid was specifically owing to interaction with the fluid of a male adopting a different tactic or more generally to the interaction with the fluid of any rival male.

In a second experiment, we compared the sperm velocity of territorial and sneaker males in mixed seminal fluids, a situation closer to that occurring in nature, where ejaculates compete. Sneakers’ seminal fluid was diluted 10-fold in filtered seawater before use to match the natural seminal fluid concentration of sneaker and territorial males (see §2b). Following the procedure described above, the velocity of the sperm of 24 territorial males (SL range: 13.9–19.0 cm) was measured either in its own seminal fluid or after incubation in a 1 : 1 mixture of its own seminal fluid and the diluted (1 : 10) fluid of a sneaker male. Similarly, the velocity of the sperm of 24 sneaker males (SL range: 6.2–9.4 cm) was measured after incubation in its own seminal fluid (diluted 1 : 10) and in a mixture (1 : 1) of its own (diluted 1 : 10) and the seminal fluid of a territorial male.

Moreover, on all individuals, employed in both experiments, sperm velocity was preliminarily measured also after incubation in activating solution, without any seminal fluid, and results were then compared with sperm velocity after incubation in each male’s own seminal fluid. This allowed to determine sperm velocity in the absence of any seminal fluid and to prove the general positive effect of males’ own seminal fluid (see details in the electronic supplementary material).

(ii) Effect of the interaction between sperm and seminal fluid on fertilization efficiency

Grass goby sneakers may enter the nest, releasing their ejaculates in proximity to territorial male ejaculate and to eggs [27,28]. Thus, as long as a sneaker succeeds in entering the nest, his sperm and seminal fluid mix with those of territorial males. In this scenario, fertilization tests performed on either sneakers or territorial males’ sperm in the presence of a mixture of both males’ seminal fluids may give a reliable indication of the fertilization success in a context of competition.

The effect of a mixture of territorial and sneaker males’ fluid on sperm fertilization rate was determined, in vitro, for 10 territorial (SL range: 13.1–16.6 cm) and nine sneaker males (SL range: 5.8–8.5 cm). We recorded fertilization rates of territorial male sperm after incubation in his own seminal fluid or in the mixture (1 : 1) of his own seminal fluid with that of a sneaker male (after 10-fold dilution in filtered sea water). The same procedure was applied to the sperm of sneaker males, incubated with their own seminal fluid (diluted 1 : 10) or with a mixture (1 : 1) of their own seminal fluid (diluted 1 : 10) and that of a territorial male.

(f) Statistical analyses

Fertilization data were arcsine square root-transformed prior to analysis. Data are reported as mean standard deviation (s.d.). Normality and homogeneity of variance were checked following Kolmogorov–Smirnov and Bartlett’s tests, respectively. Effect of treatment (seminal fluid) on performance and fertilization rate of territorials’ and sneakers’ sperm were analysed using a univariate ANOVA for repeated measure (generalized linear model). The treatment with different seminal fluids was used as within-subject factor, and the male tactic as between-subject factor. Post hoc comparisons of interest were performed through t-test for independent samples when comparing treatments between groups, and through paired t-test when comparing treatment within groups. p-values were adjusted for multiple testing following Benjamini and Hochberg method. Statistical tests were performed using STATISTICA v. 7.0. Data have been deposited in the Dryad Repository [34].

3. Results

(a) Effect of seminal fluid on sperm performance

The results of the first experiment, where sperm experienced both own seminal fluid and that of a male performing the same or a different tactic, showed a significant effect of treatment on sperm velocity, in opposite directions depending on the adopted tactic (repeated measures ANOVA: male tactic, \( F_{1,31} = 0.70, p = 0.41, \) treatment, \( F_{2,62} = 5.14, p = 0.008; \) tactic × treatment, \( F_{2,62} = 3.60, p = 0.033)\). Indeed, the sperm of sneaker males proved faster when incubated in territorial males’ fluid than in their own (paired t-test: \( t = −2.9, \) adjusted \( p = 0.01; \) figure 1a), while territorial males’ sperm proved slower when exposed to sneakers’ fluid than when incubated in their own fluid (paired t-test: \( t = 2.97, \) adjusted \( p = 0.01; \) figure 1a). This reduced performance of territorial males’ sperm cannot be ascribed to the dilution of their seminal fluid in the mixed tactic treatment (see details in the electronic supplementary material). A between-tactics comparison showed that sneakers’ sperm in territorial fluid performed better than territorial males’ sperm in sneakers’ fluid (t-test: \( t = −2.7, \) adjusted \( p = 0.01; \) figure 1a). Moreover, the velocity of both territorial and sneakers’ sperm was not affected by the seminal fluid of a male adopting the same tactic (paired t-test: sneakers, \( t = 0.45, p = 0.66, n = 20; \) territorial, \( t = 1.4, p = 0.18, n = 13),\) suggesting that the effect of seminal fluid on sperm performance is tactic-dependent and not due to the interaction with non-self seminal fluid.

In the second experiment, where seminal fluids of two male types were mixed, we again observed a significant effect on sperm velocity depending on the tactic, adopted by males (repeated measures ANOVA: male tactic, \( F_{1,46} = 2.87, p = 0.1, \) treatment, \( F_{1,46} = 0.17, p = 0.68; \) tactic × treatment, \( F_{1,46} = 23.63, p < 0.001\); figure 1b). The sperm of sneaker males were found to be faster with the addition of territorial males’ fluid (paired t-test: \( t = −2.45, \) adjusted \( p = 0.022; \) figure 1b), while territorial males’ sperm were slowed by the addition of a sneaker’s fluid (paired t-test: \( t = 6.29, \) adjusted \( p < 0.001; \) figure 1b). The between-tactics comparison confirmed the results of the first experimental set. Indeed, sneakers’ sperm exposed to own plus territorial males’ fluid swam significantly faster than territorial males’ sperm in the presence of own plus sneakers’ fluid (t-test: \( t = 3.23, \) adjusted \( p = 0.0036\); figure 1b).
diluted 1:10; white bars, own fluid; grey bars, own fluid of a male performing the same or a rival tactic ($n = 20$ territorial, 20 sneaker) while sneaker males' sperm showed significantly lower fertilization rates when sneaker males' fluid was added ($t = 2.32$, adjusted $p = 0.043$; figure 2). In particular, sneaker males' sperm showed significantly higher fertilization percentages with the addition of rival-tactic male fluid ($n = 24$ sneaker, 24 sneaker; sneaker's fluid pre-diluted 1:10; white bars, own fluid; grey bars, own + rival-tactic male fluid). *Adjusted $p < 0.05$; **adjusted $p < 0.01$; ***adjusted $p < 0.001$.

In vitro fertilization experiments showed an effect of the treatment on the fertilization efficiency of both territorial and sneaker males, in opposing directions (repeated measures ANOVA: male tactic, $F_{1,17} = 1.49$, $p = 0.24$; treatment, $F_{1,17} = 0.03$, $p = 0.87$; tactic × treatment, $F_{1,17} = 13.13$, $p = 0.002$; figure 2). In particular, sneaker males' sperm showed significantly higher fertilization percentages with the addition of rival-tactic male fluid (paired $t$-test: $t = -3.10$, adjusted $p = 0.043$; figure 2), while territorial males' sperm had significantly lower fertilization rates when sneaker males' fluid was added (paired $t$-test: $t = 2.36$, adjusted $p = 0.043$; figure 2). The between-tactics comparison showed that sneaker's sperm had a significantly higher fertilization rate in the presence of his own seminal fluid mixed with that of a territorial male, when compared with that of territorial male's sperm in the presence of his own seminal fluid mixed with that of a sneaker male ($t$-test: $t = 2.32$, adjusted $p = 0.043$; figure 2). The observed results did not change when also considering the number of eggs as a covariate in the model (repeated measures ANOVA: number of eggs, $F_{1,17} = 0.30$, $p = 0.59$; male tactic, $F_{1,17} = 1.47$, $p = 0.24$; treatment, $F_{1,17} = 0.01$, $p = 0.93$; tactic × treatment, $F_{1,17} = 12.25$, $p = 0.003$).

4. Discussion

We found that the seminal fluid differently affects the sperm performance of other males in terms of velocity and fertilization success, in relation to the tactic adopted by males. Indeed, the performance of territorial males' sperm is negatively affected by the seminal fluid of sneaker males, while sneaker's sperm perform significantly better in the presence of territorial males' seminal fluid. Thus, sneaker males, always mating in competition, enhance their fertilization success by exploiting the seminal fluid of territorial males, and decrease that of territorial males by altering their investment in seminal fluid. These results highlight that male allocate their ejaculate investment in both sperm [2,18] and non-sperm components in response to sperm competition risk, and add an important observation to the understanding of ejaculate evolution.

Grass goby ejaculates vary in both sperm number and seminal fluid amount according to the mating tactic adopted by males, with those of territorial males being richer in seminal fluid and poorer in sperm than those of sneaker males [27,28]. However, the variation in seminal fluid volume does not solely account for the entire pattern emerging from our study. Indeed, the performance of territorial and sneaker
males’ sperm in their own seminal fluid, as well as in that of other males performing the same tactic, is similar (see also the electronic supplementary material), but when the fluid of a male employing a different tactic is present, sperm performance goes in opposite directions. Moreover, in a mixture of territorial and sneaker males’ seminal fluids, sneakers’ sperm are faster than territorial males’. This suggests that: (i) the detrimental effect of sneakers seminal fluid on territorial males’ sperm is not due to a self/non-self process, but rather to a variation in composition; and (ii) the sperm released by territorial and sneaker males differ in quality. However, how the composition of seminal fluid changes with ejaculate size in males adopting ARTs and which components affect sperm velocity are still unknown.

A substantial amount of research documents the influence of different seminal fluid components in stimulating sperm capacitation, enhancing sperm speed and viability, and providing nourishment to sperm, particularly in mammals and insects (for review, see [19]). Moreover, an intra-specific variation in seminal fluid composition in relation to ejaculate size has been proposed to occur in frogs, where seminal fluid taken from the large ejaculate released by dominant males increases sperm velocity, while that of the smaller ejaculates released by subdominant males decreases it [35]. Much less is known about fish, but seminal fluid proteins with molecular weight less than 50 kDa, monosaccharides, and triglycerides seem to affect sperm viability and speed [36,37]. The seminal fluid of grass goby territorial males might be richer in these compounds to sustain the fertilizing ability of their sperm. Territorial males’ sperm are embedded in an abundant and viscous seminal fluid that, by slowly dispersing and diluting in seawater, allows a steady supply of sperm during spawns lasting, on average, 8 h [27,28]. Territorial males begin to lay their ejaculates before egg deposition, while sneakers release theirs in proximity to eggs [27]. Thus, sneakers’ sperm may exploit the seminal fluid of the territorial males’ ejaculates. Sperm of external fertilizers has to deal with hostile environmental conditions, in particular with the different water osmolarity compared with that of their seminal fluid [38], and the exploitation of rival seminal fluid could reduce the cost of ejaculate production for sneakers [25]. A similar tactic-dependent investment in non-sperm components of the ejaculate probably occurs in numerous fish species, including salmonids, blennies, wrasses, damselfishes, sunfishes, cichlids and other gobies [39], where parasitic males mate in the presence of a territorial male, and thus might exploit their seminal fluid. This expectation is supported by the observation that in those species where seminal fluid is produced by specific accessory organs, these are more developed in territorial than in opportunistic males [37,40–42].

Still, grass goby sneakers’ sperm appear to be of higher quality than those of territorial males. An increase in sperm quality in response to higher sperm competition risk has been documented in several species [8,10,15,43–46]. However, grass goby sneakers’ sperm do not differ from those of territorial males in the performance parameters commonly recorded, including morphology, velocity, viability, longevity and ATP content [10,11]. This suggests that differences in sperm quality between tactics can only be detected in the presence of a mixture of seminal fluids, an approach not considered in previous studies. It is unknown which physiological feature allows sneakers’ sperm to make the best of territorial males’ seminal fluid. Similarly, it is unknown which components of seminal fluid might be involved in impairing territorial males’ sperm and enhancing sneakers’ sperm. Both aspects need to be elucidated to understand the proximate mechanisms driving sperm–seminal fluid interaction in this species.

Regardless of the physiological mechanisms by which males alter sperm and seminal fluid quality, a general pattern can be derived from these results. Grass goby territorial males, not necessarily mating in competition, bias their ejaculate allocation to seminal fluid, having to guarantee fertilization and to limit sperm waste during the long-lasting spawning [27,28]. By contrast, sneakers, taking advantage of territorial males’ seminal fluid, lower the quality of their own fluid, and may increase the investment in sperm number [28] and quality. This may have important implications for the evolution of ejaculate composition and allocation strategies. Indeed, the outcome of sperm competition may not only be determined by sperm quality or number [47], as seminal fluid may also affect sperm performance. The importance of considering the effect of sperm–seminal fluid interactions in response to sperm competition has been stressed by theoretical studies [25,26]. Our results, in line with these expectations, underline that further experimentation to understand the evolution of ejaculate allocation strategies cannot neglect the effect of seminal fluid on sperm fertilization success.

We are grateful to A. Pilastro for valuable comments on the manuscript and to A. Sambo for the endless field assistance. Financial support was provided by Cariparo Foundation. The experiments conducted herein comply with the current Italian laws (permission CEASA University of Padova no. 35/2011).

References


